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AEROBIC HETEROTROPHIC BACTERIAL POPULATIONS OF SEWAGE AND ACTIVATED SLUDGE. II. METHOD OF CHARACTERIZATION OF ACTIVATED SLUDGE BACTERIA

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Aerobic Heterotrophic Bacterial Populations of Sewage and Activated Sludge

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The replica-plating technique and Lochhead's nutritional method were combined in exploratory experiments to test their feasibility as useful means for characterizing the aerobic heterotrophic flora of activated sludge and to minimize the burdensome process of isolation, purification, and testing of isolates. In the test run, the method was about 86% reliable at the 0.05 level of significance. About 40% of the total number of bacteria able to grow on an aqueous extract of activated sludge did not grow on media containing glucose, amino acids, growth factors, and inorganic salts. The requirement for activated sludge extract suggested the existence of a requirement for unidentified nutrients contained in the activated sludge extract

From the few attempts to characterize the bacterial populations of activated sludge, there have emerged two principal points of view: the bacteria in the flocs were normally heterogeneous, and the flocs were restricted to a few genera. As a result of the classical studies of Butterfield (2), it was believed the *Zoogloea ramigera*, a floc-forming organism was the most predominant and active organism in the stabilization of waste. Heukelekian and Littman (7) confirmed the importance of *Zoogloea*-forming bacteria in waste treatment. However, McKinney and Weichlein (11) isolated several species of bacteria belonging to different genera from activated sludge. Allen (1) identified *Flavobacter*, *Achromobacter*, and *Chromobacter* in laboratory activated sludge. In these studies, the predominant bacteria of the natural activated sludge were reported to be from water and soil in origin, with a preponderance of *Pseudomonas* species. VanGils (18) reported that *Achromobacter*, *Alcaligenes*, and *Flavobacter* were predominant in both natural and laboratory activated sludges with *Pseudomonas* and coryneform organisms occurring in relatively low numbers. Dias and Bhat (4) reported the presence of *Zoogloea* and *Comamonas* in activated sludge and stated that coliforms formed only a small portion of the total population, indicating that these organisms played a minor role in sewage purification. R. E. Anderson (Ph. D. Thesis, Univ. of Wisconsin, Madison, 1964) found activated sludge to be heterogeneous in population and attached greater significance in the nonfloc-forming bacteria in the purification of sewage than has been previously recognized.

Previously, most workers relied on conventional rather than available methods. The extinction dilution method was used to estimate numbers of different physiological types with appropriate substrates, and random isolates were subcultured, tested, and identified if possible. A serious drawback in the differential enumeration of bacterial types is the difficulty, or even the impossibility, of precise correlation of the numbers and types of bacteria obtained by plating separate samples from the same population on several selective or differential culture media, both because of sampling errors in obtaining the inoculum and because of the overlapping categories which result in certain of the generally used categories; e.g., aerobes, proteolytic organisms, acid formers, nitrate reducers, ammonifiers are classes not likely to be mutually exclusive. When isolates are studied, the number of strains and subcultures to be examined quickly become excessively burdensome, and, furthermore, it is unlikely that most of the isolates will be identifiable, except in broad terms, from available information.

In attacking the problem of the bacteriology of

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activated sludge, we chose to simplify the work by restricting ourselves to the aerobic heterotrophs, since these fulfill, as a group, the function of degrading the organic materials. We were then able to adopt the nutritional categories of Lochhead and Chase (10) for describing the population structure. These categories divide the population according to the complexity of nutritive requirements, and it is then unnecessary to rely on taxonomic categories for quantitative characterization of the populations. Once the means of ascertaining the population structure has been attained, the changes under the influence of stimuli can be used to deduce information as to the adaptability of the population.

To use the method as it was employed by Lochhead, it was necessary to find a medium analogous to the soil extract medium which would support as many activated sludge bacteria as possible. An activated sludge extract (ASE) agar was found to be a consistently highly productive medium for enumeration of activated sludge bacteria (13).

As a means of eliminating some of the labor of isolation and subsequent testing and of reducing some of the sampling error inherent in the use of different samples, the replica plate technique of Lederberg and Lederberg (9) seemed especially suitable for simplifying the analysis of mixed populations. By combining this technique with Lochhead's scheme of nutritional analysis, colonies growing on the surface of a master plate of "nonselective" medium, in our case ASE agar, can be transferred to a series of other plates of increasing nutritional complexity and media of other compositions as desired. Replicated colonies of bacteria develop according to their capabilities in the same relative positions as on the master plate, and the properties of the replicates can be ascribed to a specific colony on the master plate.

Lechevalier and Corke (8) used the replica plate technique for rapid identification of antibiotic producing actinomycetes. Wiseman and Sarles (20) also employed it for identifying and enumerating the coliforms of chickens fed on antibiotic-supplemented feed. Runnels and Wilson (16) used the technique in the isolation and study of staphylococci from the nostrils of animals and humans. In a study of toxicity of metals and plastics to mixed cultures of bacteria, Rosenwald et al. (14) used the replica plate method. Modified versions of this technique have also been reported (3, 12).

Although the replica plate technique has previously been used successfully for other purposes, its application for characterizing quantitatively a heterogeneous population like that of activated sludge requires evaluation because of several inherent complications. There is the possibility that the texture of some colonies on the master plate may prevent transfer of the colonies. In contrast, some colonies may be depleted so that nothing of the colony is left on the velveteen at some subsequent stage of the transfer series. Furthermore, though the colonies may appear to be pure, some of them may actually be impure. Interaction between the neighboring colonies through syntrophism or antagonism can also give misleading nutritional results, elevated counts, or repression of colonies. These effects are detected or avoided when the organisms are isolated in pure culture. Colony size influences the reproduction of colony form. Smearing of reproduced colonies may occur, but it is not a serious limitation, because the large colonies which are the cause are seldom encountered on the ASE master plate. Barring significant interference from the aforementioned factors, the use of the replica plate technique for the transfer of colonies of activated sludge bacteria randomly distributed on plates of ASE agar onto plates containing various indicator media should facilitate characterization of the population of aerobic heterotrophs of activated sludge. This report describes the results of a comparison of the nutritional characterization of the activated sludge bacteria as studied by conventional streak plating and isolation and by the replica plate method, showing the feasibility and limitations of the latter.

MATERIALS AND METHODS

The method of replica plating, as described by Lederberg and Lederberg (9) was followed.

For preliminary tests with mixtures of pure cultures, the plating medium principally used was Casitone glycerol yeast autolysate agar (CGY) containing 5 g of Casitone, 5 g of glycerol, 1 g of yeast autolysate, and 1,000 ml of distilled water. The *p*H of the medium was adjusted to 7.2.

The media for the determination of nutritional requirements (Table 3) were prepared as described by Rouatt and Lochhead (15).

Freshly collected activated sludge from a treatment plant was used for experimental population studies. Aqueous extracts of activated sludge (ASE) were prepared as described previously (13).

Unless otherwise indicated, agar plates were inoculated by surface spreading with a bent glass rod.

RESULTS AND DISCUSSION

Preliminary experiments were made with artificial mixtures of bacteria which formed butyrous colonies to make a rigorous evaluation of the probability of occurrence of mixed colonies. The mixture contained approximately equal

numbers of *Escherichia coli*, *Pseudomonas fluorescens*, and an unidentified red-pigmented bacterium isolated from activated sludge. Experiments were also run to discover evidence of suppression of numbers or of colony size and to determine whether the concentration of nutrients had any effect. For brevity, only the conclusions will be reported here. In replication experiments, 100% of the colonies were replicated without excessive smearing, when the master and replicated plates were 0.1 to 0.04 of full concentration of CGY. More than 95% of the replicated colonies were pure, as proven by reisolation. There was no evidence of interference between strains on countable plates as seen by absence of significant deviation between actual and expected counts. It was concluded that conditions favoring small, well-separated colonies were optimal for replica plating and that mixed colonies would be at sufficiently low levels to be tolerable.

Further experimental studies were conducted with activated sludge to investigate the following factors likely to limit the applicability of the replica plate technique for characterization of activated sludge bacteria.

(i) *Failure of transfer of colonies grown on ASE plates.* To evaluate the effects of failure of transfer, two ASE master plates which had been incubated at 28 C for 10 days after inoculation with dilutions of activated sludge were replicated, each on a set of five plates of ASE agar, and were incubated at 28 C. After 10 days, the replicated plates were compared with the corresponding master plates.

The confidence limit, computed around the grand mean of the per cent number of colonies replicated (Table 1), by use of a single tail test and 0.05 level of significance (5), indicated that a minimum of 93% of the colonies on the master plate can be transferred onto a series of five replicates.

TABLE 1. *Replication of colonies grown on activated sludge extract agar*

Replication no.	Master plate I (176 colonies)[a]		Master plate II (73 colonies)	
	No. of colonies replicated	Per cent replicated	No. of colonies replicated	Per cent replicated
1	168	95.5	72	98.7
2	167	95.2	71	97.4
3	165	93.9	70	96.0
4	163	92.8	70	96.0
5	160	91.0	70	96.0
Total	823		353	

[a] Per cent weighted mean = 823/880 × 100 = 93.7; variance = 3.41; and grand mean = 95.2.
[b] Per cent weighted mean = 353/365 × 100 = 96.8, and variance = 1.47.

TABLE 2. *Mixed colonies on activated sludge extract agar master plates*

Plate no.	Total no. of colonies	No. of mixed colonies (observed)	Per cent mixed in total
1	47	3	6.4
2	33	3	9.0
3	149	13	8.7

TABLE 3. *Discrepancies in replica plating*

Medium (Lochhead series)	Total no. of colonies on master plate	Total no. accounted for	No. of discrepancies	Matching colonies (%)
Basal medium	127	126	1	99.3
Basal medium + yeast extract	127	118	9	92.1
Basal medium + yeast extract + vitamin B_{12}	127	116	11	91.4
Basal medium + amino acids	127	116	11	91.4
Basal medium + amino acids + growth factors	127	116	11	91.4[a]

[a] Reliability of replication: ~~91.4~~ − 1.96 (0.086 × 0.914)$^{0.5}$ = 86%, at 0.05 level of significance.

(ii) *Estimate of error due to mixed colonies.* Inocula of activated sludge were plated on three ASE agar plates. All the colonies developing after 7 days were streaked on sterile plates of ASE agar to test their purity. Table 2 represents the results obtained.

The probability of the occurrence of mixed colonies can be computed as a confidence limit around the per cent weighted mean of mixed colonies found on the ASE master plates. The confidence limit computed from the data in Table 2 is 8.3 ± 3.5%, at 0.05 level of significance, which indicated that a range of 5 to 12% of mixed colonies was to be expected. Such an incidence was considered tolerable.

Application of replica plating to nutritional characterization of activated sludge bacteria. Activated sludge was plated on ASE agar in a plastic petri dish. Colonies developing on this master plate were identified by numbers etched with a metal stylus on the bottom of the petri dish. Before replica plating, inocula were first picked with a needle from all the colonies on this plate and suspended separately in about 1 ml of

sterile distilled water. The colonies on the same master plate were then replicated on the agar media of Lochhead's nutritional series in the following order: basal medium, basal medium + yeast extract, basal medium + yeast extract + vitamin B_{12}, basal medium + amino acids, and basal medium + amino acids + growth factors.

A loopful of each prepared suspension of cells previously picked from the colonies was streaked on plates of the above agar series. All the plates, i.e., the replica plates and the streaked plates, were then incubated at 28 C for 10 days, and scores were given as + or −, depending upon whether there was growth (+) or no growth (−) for each colony on each medium. In this way, isolates were obtained, purified, and separately tested for ability to grow on various substrates. A comparison of the replica plate method and streak plate method should indicate within fiduciary limits whether the replica plate method is quantitatively as valid a method for characterization of heterogeneous populations as the method of isolation and testing pure cultures. The growth responses of all 127 colonies grown on an ASE master plate on the five Lochhead media as obtained by the replica plate method were compared with the growth responses of the purified isolates on the same media. When there was a difference between responses of the replicated colony and the pure culture, a discrepancy was recorded regardless of which member of the pair failed to grow. Table 3 shows the number of discrepancies, i.e., the number of colonies that showed opposite responses (growth or no growth) for each medium.

TABLE 4. *Comparative evaluation of nutritional characterization of activated sludge bacteria by replication versus isolation*[a]

Medium	Replica plate method		Streak plate method	
	Colonies grown	Per cent[b] grown	Colonies grown	Per cent[b] grown
Basal medium	49	38.5	50	39.3
Basal medium + yeast extract	56	44.1	65	51.2
Basal medium + yeast extract + vitamin B_{12}	59	46.4	70	55.1
Basal medium + amino acids[c]	62	48.8	73	57.5
Basal medium + amino acids + growth factors[d]	62	48.8	73	57.5

[a] Total number of colonies on master plate = 127; number of colonies that did not grow on any of the replica plates = 65; and number of colonies that did not grow on any of the streak plates = 54.

[b] Per cent of the total number of bacteria on ASE plate.

[c] Vitamine-free Casamino Acids (Difco).

[d] Cysteine, thiamine, biotin, pyridoxine, pantothenic acid, nicotinic acid, riboflavine, and inositol.

TABLE 5. *Distribution of activated sludge bacteria by nutritional categories*

Nutritional group	Replica plate method[a,b]	Streak plate method[a,b]
(1) Requiring ASE	51.2	42.5
(2) Requiring basal medium	38.5	39.3
(3) Requiring yeast extract	5.6	11.9
(4) Requiring vitamin B_{12}	2.3	3.9
(5) Requiring amino acids	10.3[b]	18.2[b]
(a) Requiring exclusively one or more amino acids	2.4	2.4
(b) Substituting for the amino acids requirement by yeast extract and vitamin B_{12}	7.9	15.8

[a] Per cent of 127, the total number of bacteria on ASE master plate. In totaling the percentages, category 5a and 5b are omitted because they contain groups 3 and 4. For the replica plate method, these percentages are 10.3 and 7.9, and for the streak plate method, 18.2 and 15.8, respectively.

[b] Group 5 comprises two subgroups, 5a and 5b, and represents their total percentages.

The number of discrepancies seems to rise during the initial steps of replication and stabilizes in the latter ones (Table 3). To evaluate the precision of the replica plate technique in such a situation, it is desirable to set a confidence limit based upon the results obtained in the last step of the replication process, which is a more rigorous limit in that it takes into account the discrepancies produced by preceding steps. The results of this study showed that the replica plate method is reliable to about 86%, at 0.05 level of significance. Discrepancies arising in this series also included whatever discrepancies might be due to nutritionally mixed (i.e., impure) colonies.

Nutritional characterization of activated sludge bacteria. The growth responses of the colonies transferred by replica plating onto the Lochhead's media in the previous experiment were compared with the growth responses of the same colonies subjected to streaking on plates containing media of the same nutritional class. Table 4 represents a

comparative evaluation of the qualitative nutritional characterization of activated sludge bacteria on Lochhead media as done by replica plating and streak plating.

The higher growth response by the streak plate method was not altogether unexpected. It was partly due to the failure of transfer of some colonies from the master plate to the replica plates. One significant observation made in this experiment was that a substantial proportion of the total population from ASE master plates, about 42%, from streak plating and about 51%, from replica plating, failed to grow on any of the media used in this nutritional characterization study. However, 97%, of the activated sludge bacteria growing on the ASE master plate, grew when reinoculated on ASE agar. This suggests that there are factors in the activated sludge not supplied in the culture media which are required for the growth of these bacteria. Although a vitamin requirement category cannot be calculated from the data in Table 4, the ASE requiring bacteria would not grow on the basal medium plus amino acids plus growth factors.

The distribution of bacteria into the nutritional categories by the replica plating method has been shown to give a valid classification according to the nutritional requirements of the organisms and to be sufficiently quantitative to equal the precision of plate counting from soil or water samples. The experiments described in this report were primarily designed as a test of validity of the technique and do not give the best results for probing the structure of activated sludge, it being necessary in these particular experiments to curtail the work to practical limits; however, by subtraction of the proper control value, the distribution of types (Table 5) was found by the replica plate and streak plate methods.

In making tentative comparisons with data reported on soil by Wallace and Lochhead (19) and on marine bacteria by Skerman (17), we found that the activated sludge population had a high incidence of bacteria utilizing the simple basal medium in common with the rhizosphere soil (about 35%). There was also a high proportion of bacteria requiring ASE in a class somewhat analogous to the bacteria in the control soil (about 30%). Soil extract, however, cannot be substituted for ASE in the enumeration of activated sludge bacteria (13). The proportion of strains requiring amino acids was similar to that in the rhizosphere of older plants, but far less than that in the marine populations. At present, such results are difficult to interpret. Many observations under different conditions will have to be recorded before generalizations as to the bacterial composition of activated sludges will be valid.

It was previously reported that extracts made from different activated sludges differed in their abilities to support high colony counts with a particular activated sludge (13), but the effect on the individual nutritional classes was not obtained. Activated sludges have been reported to behave differently (6), but the causes are not presently understood. The variables of operation of the treatment plant and the quality of the waste apparently affect both the composition of the population indigenous to the activated sludge and the composition of the ASE.

At this stage of research, the assessment of the comparative nutritional deficiencies of the extracts is somewhat a circular problem, especially with regard to an ill-defined requirement for ASE, since we do not at present know whether the basis of the requirement is for a specific growth factor, for multiple factors, or for some physical or chemical condition of the culture medium, nor do we have much information as to the other nutritional requirements of this class of microorganisms.

It should be understood that the experiments and methods herein reported do not represent the ultimate refinements or the limits of possibilities of the application of the method. Experiments of a similar nature, but in a somewhat different context, will be reported elsewhere.

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